In The Name Of God

Effect of dacarbazine on live melanoma cells as measured by atomic force microscopy based nanoscopy

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List of contents

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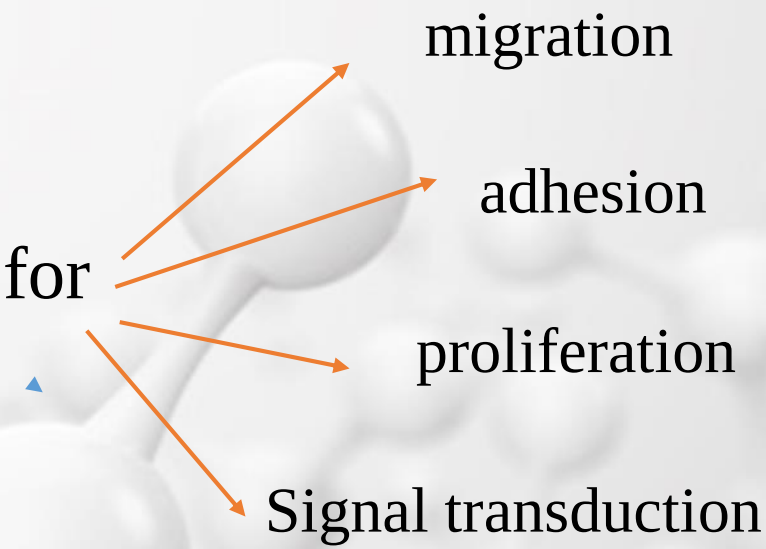
Abstract:

- CD44 ligand-receptor interactions are known to be involved in regulating cell migration and tumor cell metastasis.
- High expression levels of CD44 correlate with poor prognosis of melanoma patients.
- The effect of DTIC treatment was quantified by the dynamic binding force and the ligand-binding free-energy landscape.
- Atomic force microscopy-based nanoscopic methods offer thermodynamic and kinetic insight into the effect of DTIC on the CD44 ligand-binding process.

Introduction:

- CD44 ligand-receptor is important for



- Melanoma cells proliferation ,tumor progression , and metastasis are mediated by the interaction of CD44 ligand-receptor.

- Increased levels of CD44s &CD44v have been considered prognostic markers for disease progression.

What is the effect if chemotherapeutic drug on CD44 expression?

- The alkylating agent dacarbazine (DTIC) is a major chemotherapeutic drug for the treatment of malignant melanoma but its efficacy is very disappointing
- the patients treated with DTIC, the tumor readily becomes resistant to the drug.

Thus

Quantitative investigation of how and to what extent DTIC influences the binding strength of CD44 ligand –receptor may improve drug treatment efficacy.

- In this investigation , high resolution AFM imaging and nanoidentation-based soft colloidal force spectroscopy were used to detect real-time changes in melanoma cell morphology , nanostructure , nanomechanical properties before and after DTIC treatment.
- This was accomplished at the single-molecule level.
- All morphology images were obtained using AFM in contact mode.

Material and methods:

CCK-8 assay to analyze cell viability

B16-F10 melanoma cells were purchased from ATCC (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, containing 10% fetal bovine serum (FBS). Before cells were seeded into 96-well plates, we determined cell viability primarily by trypan blue staining and determined the number of cells by cell counting. The percentage of viable cells was about 99% and the cell number was about 3×10^4 cells/mL. Cells in exponential growth were placed in 96-well plates at a final concentration of 3×10^4 cells/mL and then treated with differing concentrations of DTIC for differing periods of time. Cell viability was assessed using the Cell Counting Kit (CCK-8) assay (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. The absorbance of each well was read at 490 nm with MK3 Absorbance Microplate Reader (Thermo Electron Corporation, Beverly, MA, USA). All data were derived from three independent experiments.

Results 1:

- The cytotoxicity of DTIC to the melanoma cell line B16-F10 was determined by CCK-8 assay.
- The IC50 value was 133.75 microgram per milliliter.

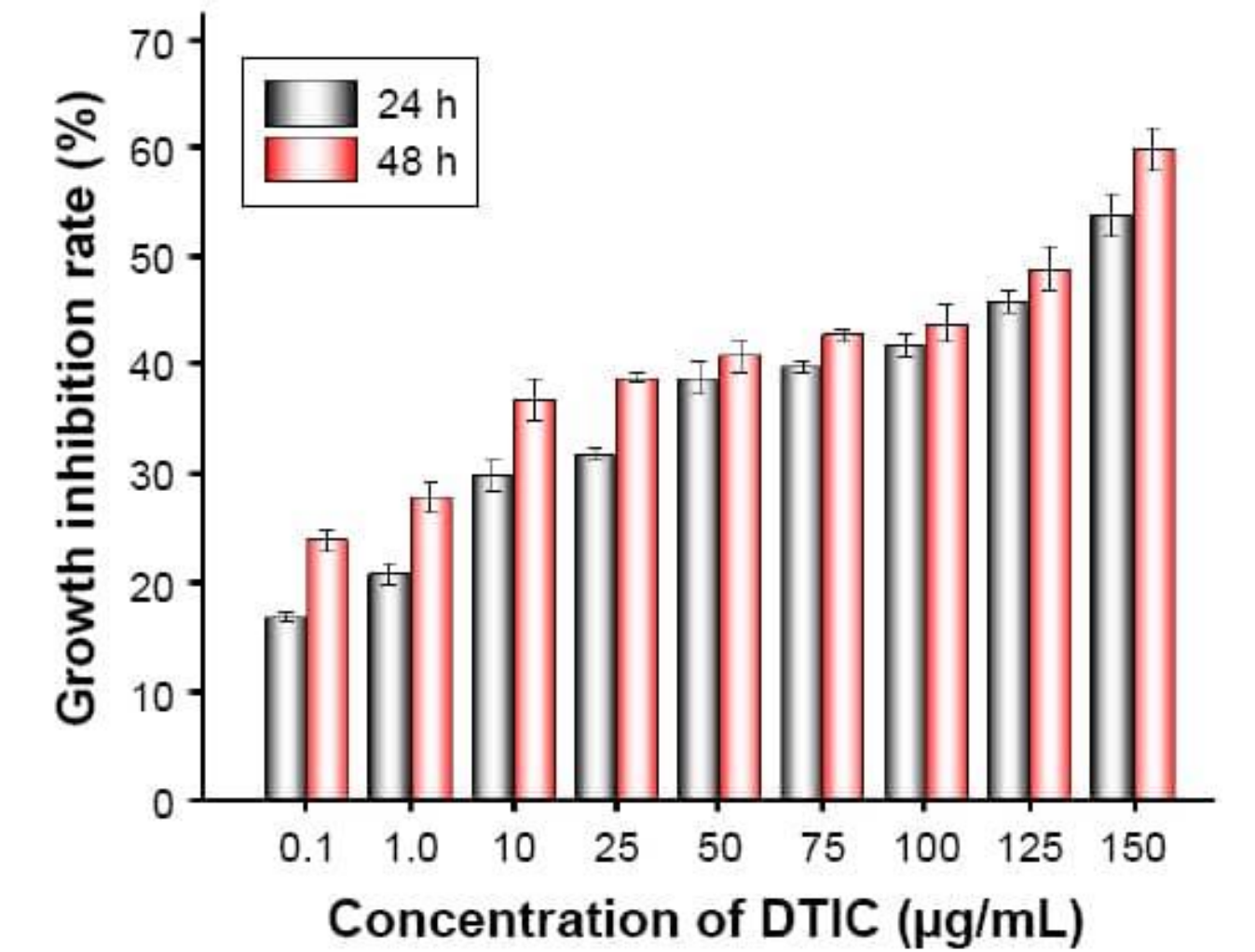


Figure S1 Inhibitory effects on the growth of B16-F10 cells following DTIC (0.1, 1.0, 10, 25, 50, 75, 100, 125, and 150 μg/mL) treatment for 24, and 48 hours.
Abbreviation: DTIC, dacarbazine.

CD44 analysis by flow cytometry (FCM)

B16-F10 cells were placed in 24-well plates at a density of 2×10^6 cells per well in 200 mL of DMEM with 10% FBS. After 6 hours, DTIC was added to the cells at differing concentrations (0, 0.1, 1.0, 10, 25, 50, 75, and 100 µg/mL). After incubation for 24 hours, fluorochrome-conjugated anti-CD44 antibody (eBioscience, San Diego, CA, USA) was added to each well. Then, the cells were analyzed using FCM (FACS Calibur; Becton Dickinson, San Jose, CA, USA). FCM results were analyzed using Cell Quest software (Win MDI; Becton Dickinson).

Antibody-conjugated quantum dot (QD) fluorescence staining

The staining procedure for the CD44 antibody and QD conjugates was as follows: control cells and the cells treated with 25, 50, and 75 μg/mL DTIC for 24 hours were washed with phosphate-buffered saline (PBS), then fixed with 2% formaldehyde at 4°C for 30 minutes, and then washed three times with PBS before staining. The cells were then blocked for 30 minutes with 1% bovine serum albumin (BSA) and incubated with 20 mg/mL biotinylated anti-mouse CD44 antibody for 60 minutes; next, the cells were incubated with 1 μg/mL QD-streptavidin for 60 minutes in the dark. CD44 molecules were viewed using a LCM 510 Meta Duo laser scanning confocal microscope (LSCM) (Carl Zeiss Meditec AG, Jena, Germany).

Immunofluorescence imaging of the cytoskeleton proteins , F-actin and tubulin

The cytoskeletal structures of B16-F10 cells treated with differing concentrations of DTIC were visualized by staining with phalloidin-fluorescein isothiocyanate (FITC) and Tubulin-Tracker. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and incubated in phalloidin-FITC or Tubulin-Tracker solution (Beyotime Biotechnology) at a dilution of 1:40 for 60 minutes in the dark at room temperature. After being washed three times with PBS, the cells were incubated with a 4′,6-diamidino-2-phenylindole (Beyotime Biotechnology) solution at a dilution of 1:200 for 15 minutes at room temperature and then washed three times with PBS. The cytoskeleton organization and cell nucleus were imaged using LSCM. The images were processed using Zeiss LSCM software.

Result 2:

- The quantitatively investigate the effect of DTIC on CD44 expression.
- FCM was used to measure the levels of CD44 by B16-F10 cells with and without DITC treatment.
- LSCM images were used to visualized the expression of CD44 on cells with or without DITC treatment.
- These data suggest that DTIC inhibits the expression of CD44 with correspondence to changes in cell morphology.

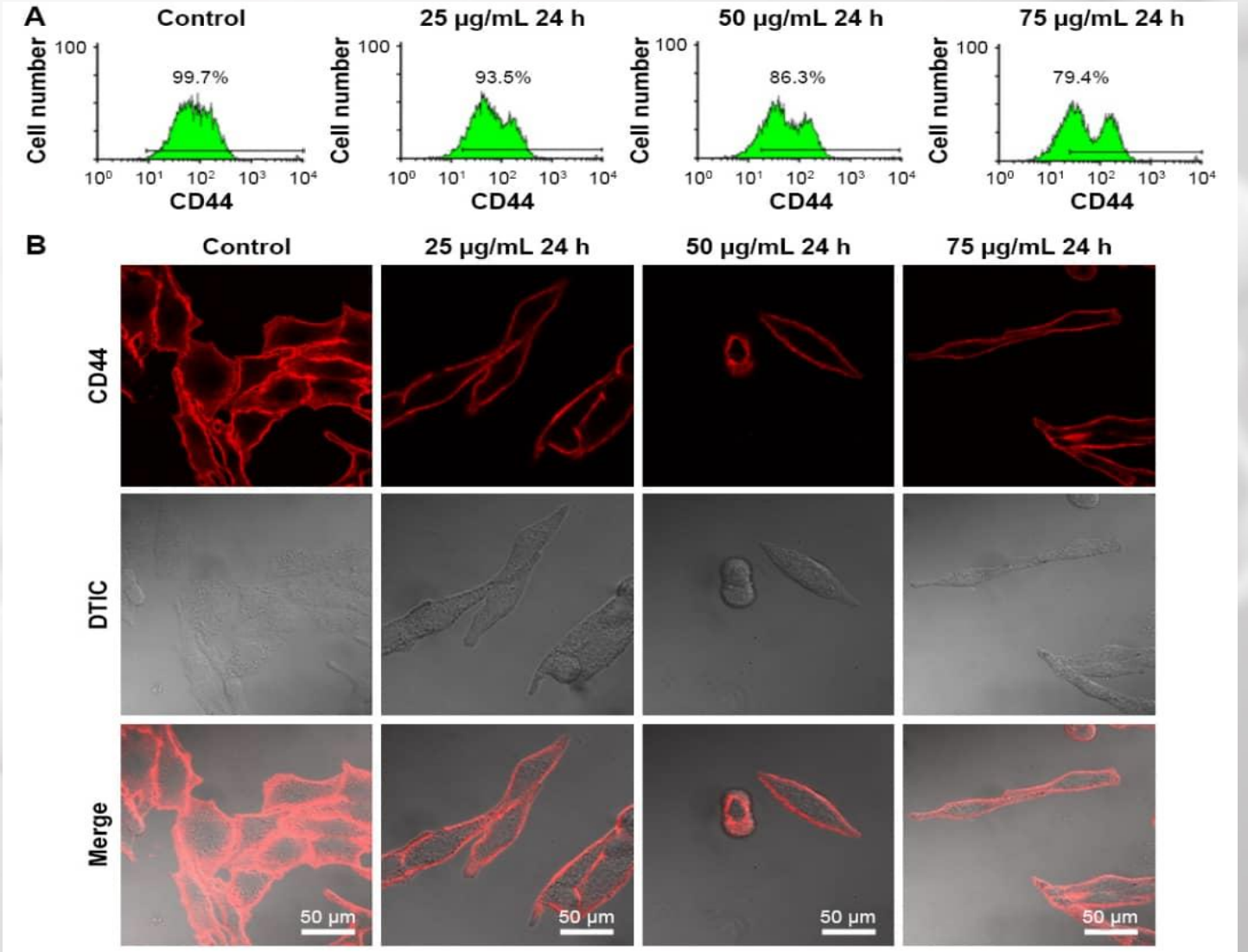


Figure 1 Qualitative and quantitative analysis of the expression of CD44 proteins in B16-F10 cells before and after treatment with DTIC.
Notes: (**A**) Flow cytometric quantitative analysis of the expression of CD44 in B16-F10 cells without DTIC treatment and in cells treated with 25, 50, and 75 μg/mL DTIC for 24 hours. (**B**) Confocal fluorescence images of the CD44 distribution in B16-F10 cells treated without DTIC and in cells treated with 25, 50, and 75 μg/mL DTIC for 24 hours.
Abbreviation: DTIC, dacarbazine.

Result 3:

- The CD44 is an organizer of the cortical actin skeleton that extracellularly binds to ligands in the ECM , and intracellularly , is mechanically linked to the actin cytoskeleton through erzin/radixin /moesin proteins.

- Signal transduction requires the cytoplasmic tail region of CD44 to interact with actin to link to the local cytoskeleton organization.

The investigation of the interaction CD44 and B16-F10 cytoskeleton before & after cell treatment with DTIC may contribute to an understanding of the treatment effect of DTIC on melanoma.

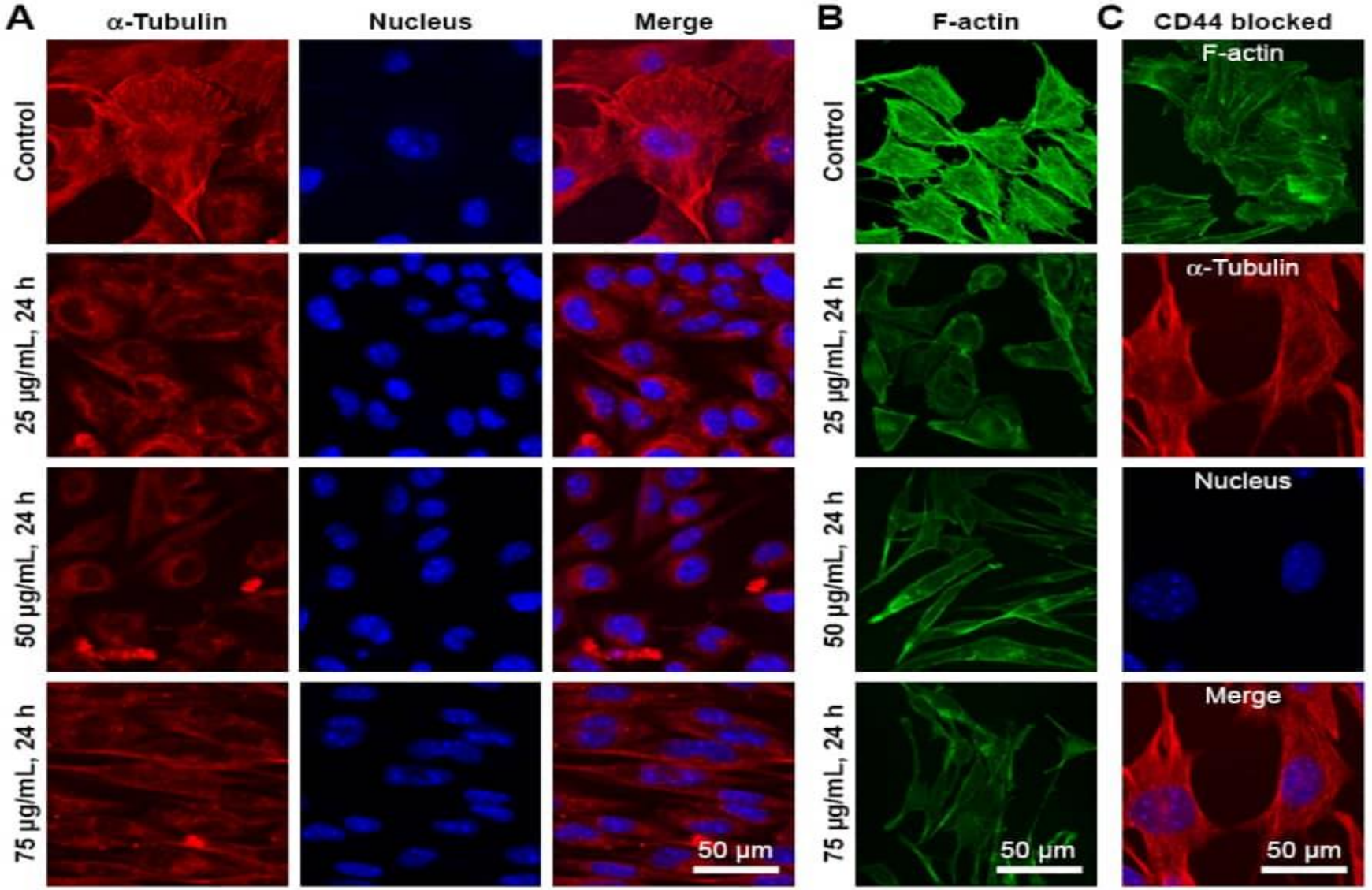


Figure 2 Organization of the cytoskeleton in B16-F10 cells.
Notes: (**A**, **B**) α-Tubulin and F-actin in cells treated without DTIC and in cells treated with 25, 50, and 75 μg/mL DTIC for 24 hours. (**C**) α-Tubulin and F-actin in cells pretreated with CD44 antibodies and then treated with 50 μg/mL DTIC for 24 hours.
Abbreviation: DTIC, dacarbazine.

AFM measurements:

- All morphology images were obtained using AFM in contact mode .
- Real-time changes were investigated in the surface morphology and ultrafine structure of B16-F10 cells treated with DTIC using AFM.
- The cell membrane is an important regulator of cell function and changes in it can directly influence the normal functions of cells.
- The AFM images revealed remarkable differences in cell morphology and surface structure in presence and absence of DTIC.

Changes in the morphological ultrastructure of a single B16-F12 cell following DTIC treatment

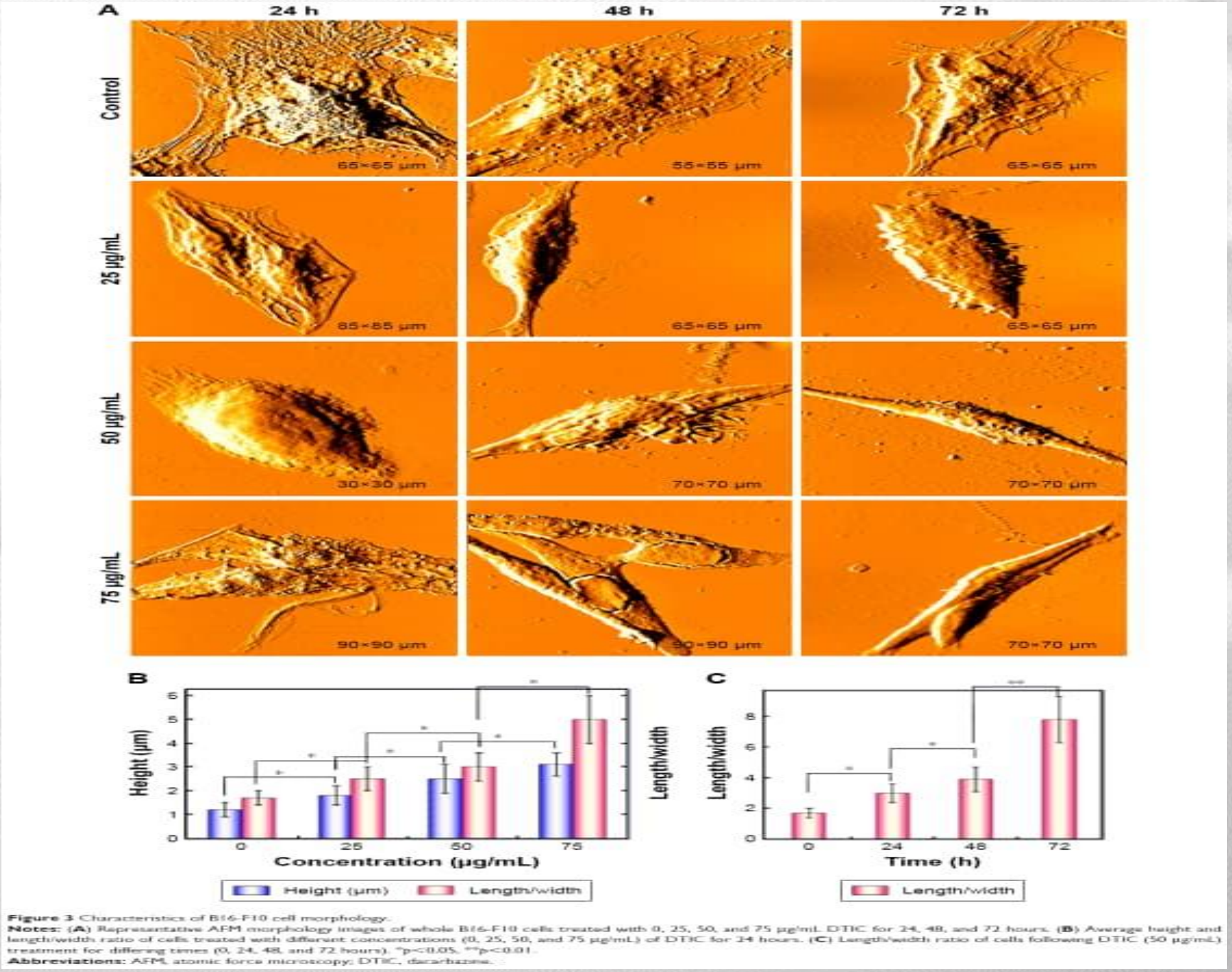


Figure 3 Characteristics of B16-F10 cell morphology.
Notes: (**A**) Representative AFM morphology images of whole B16-F10 cells treated with 0, 25, 50, and 75 μg/mL DTIC for 24, 48, and 72 hours. (**B**) Average height and length/width ratio of cells treated with different concentrations (0, 25, 50, and 75 μg/mL) of DTIC for 24 hours. (**C**) Length/width ratio of cells following DTIC (50 μg/mL) treatment for differing times (0, 24, 48, and 72 hours). *p<0.05, **p<0.01.
Abbreviations: AFM, atomic force microscopy; DTIC, dacarbazine.

As shown in this figure , cell membrane were damaged and showed some umblications and particles enlarged and were heterogonous.

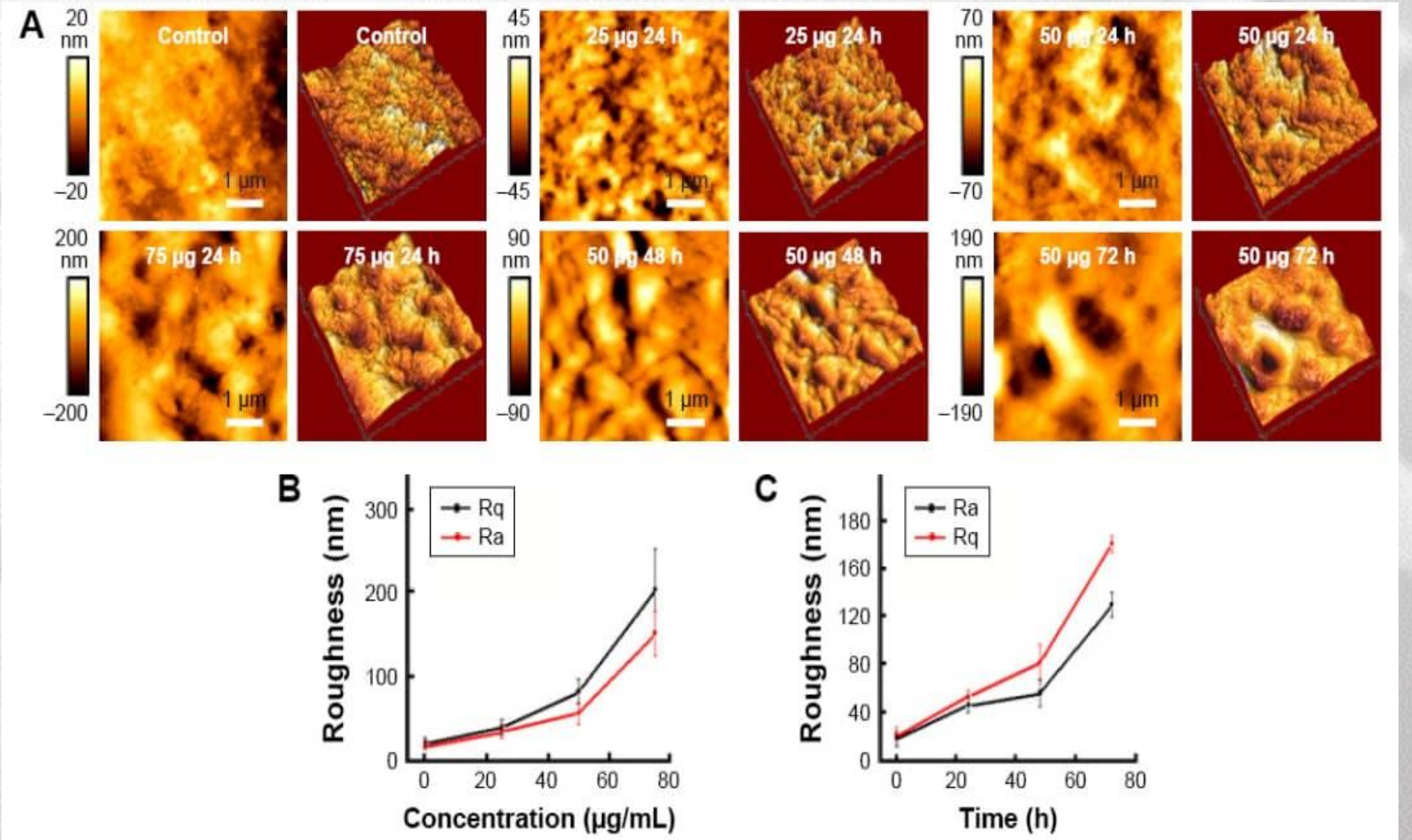


Figure 4 Characteristics of B16-F10 cell membrane ultrastructure.

Notes: (**A**) Changes in B16-F10 cell membrane ultrastructure after treatment with 0, 25, 50, and 75 μg/mL DTIC for 24, 48, and 72 hours. (**B**, **C**) The surface roughness parameters Ra and Rq of cells treated with different concentrations (0, 25, 50, and 75 μg/mL) of DTIC for 24 hours and of cells following DTIC (50 μg/mL) treatment for different times (0, 24, 48, and 72 hours).

Abbreviations: DTIC, dacarbazine; Ra, average roughness; Rq, root-mean-square roughness.

In order to quantify the fraction of cell membrane damaging induced by DTIC, release of lactate dehydrogenase (LDH) into the medium was measured.

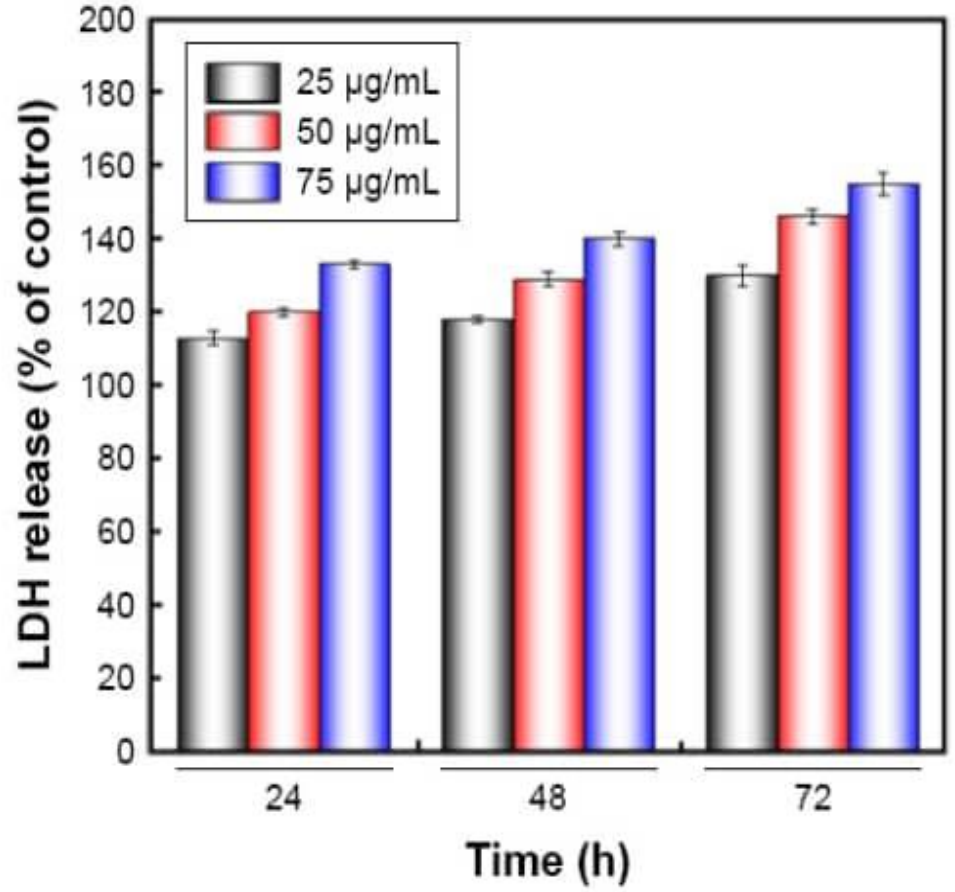


Figure S3 LDH assay to quantify the fraction of cell membrane damaging about B16-F10 cells following DTIC (25, 50, and 75 µg/mL) treatment for 24, 48, and 72 hours, respectively.
Abbreviations: DTIC, dacarbazine; LDH, lactate dehydrogenase.

Alterations in the nano-biomechanical properties of cells treated with DTIC

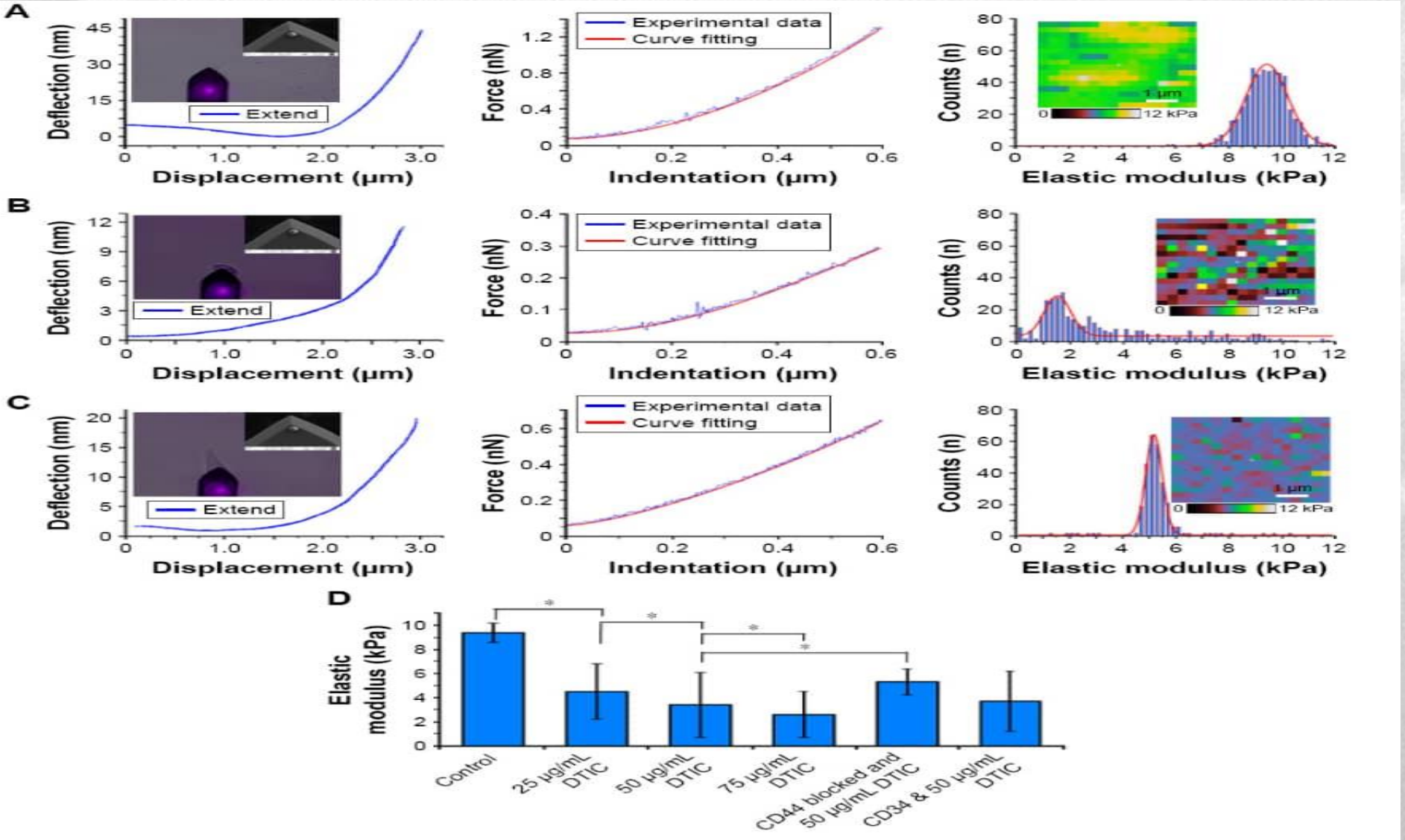


Figure 5 Alterations in the nano-biomechanical properties of B16-F10 cells treated with DTIC, as detected by AFM force spectroscopy.
Notes: Representative AFM deflection–displacement curves, force–indentation curves (blue lines), curve-fitting based on the Hertz model (red lines), histogram of the elastic modulus, and elasticity map of: control cells (**A**), cells treated with 50 μg/mL DTIC for 24 hours (**B**), and cells pretreated with CD44 antibodies and then treated with 50 μg/mL DTIC for 24 hours (**C**), and (**D**) histograms of the elastic modulus of the cells treated with different concentrations of DTIC for 24 hours. *$p<0.05$.
Abbreviations: AFM, atomic force microscopy; DTIC, dacarbazine.

So DTIC:

Inhibited the expression of CD44

Disrupted the local linkage of CD44 to the cytoskeleton

Triggering a decrease in cellular elasticity

AFM nanoscale imaging of CD44 molecules using single-molecule force spectroscopy(SMFS)

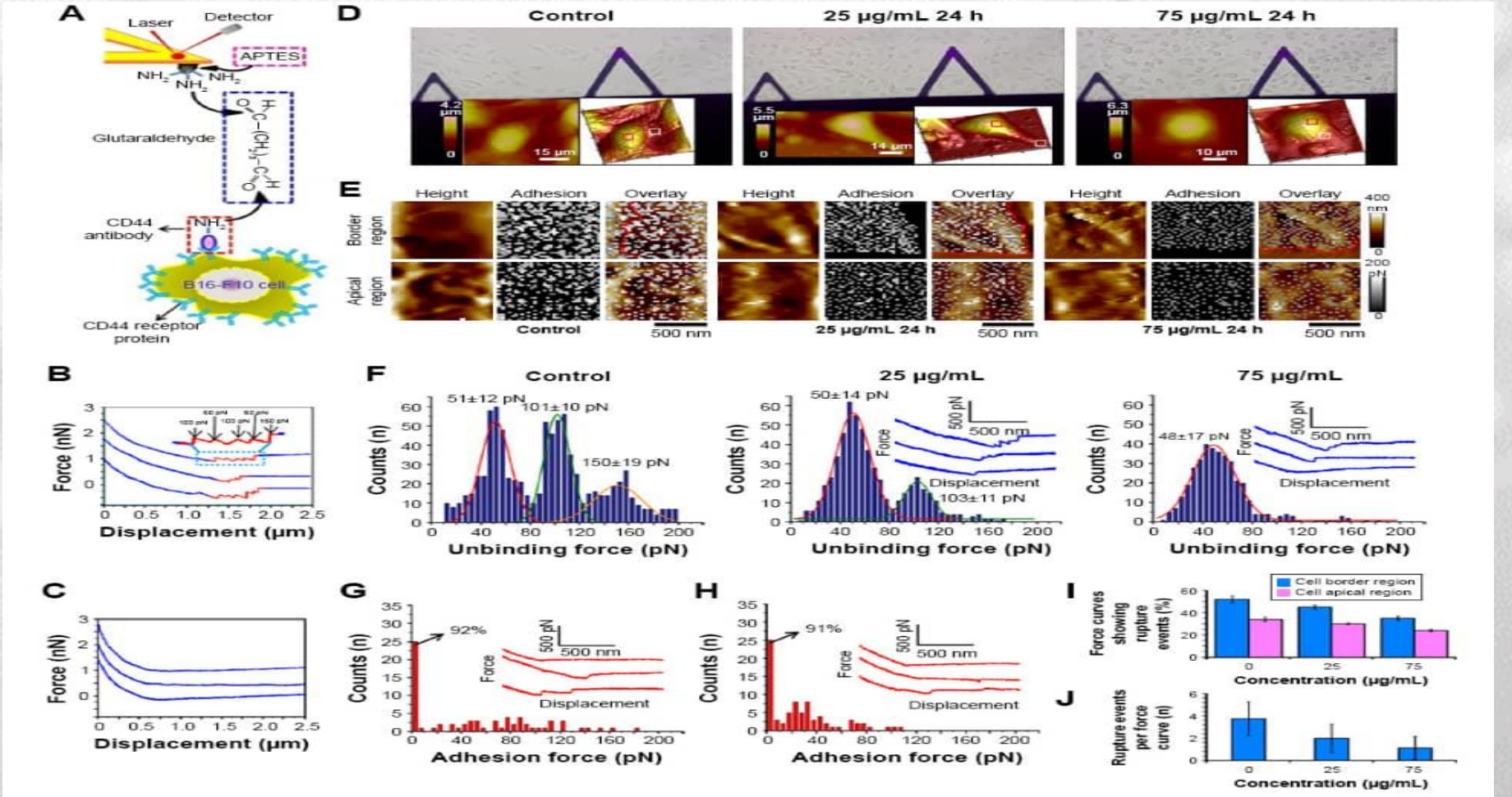


Figure 6 The specific interaction between CD44 and living B16-F10 cells, as investigated by AFM force spectroscopy and force mapping.
Notes: (**A**) Schematic representation of the strategy used for the immobilization of CD44 onto the AFM tip. (**B** and **C**) Representative force curves recorded with CD44-functionalized tip and bare tip on control cells. (**D**) AFM images of living cells treated without DTIC and with 25 and 75 μg/mL DTIC for 24 hours. (**E**) AFM topography images, CD44 recognition image of the B16-F10 cell surface using a CD44 antibody-functionalized AFM tip. (**F**) Histograms of CD44$^+$ live cell specific unbinding forces and typical force curves. (**G**) Force curves and adhesion force histogram obtained after CD44 binding sites were blocked. (**H**) Force curves and histograms obtained using a BSA tip. (**I, J**) Percentage of force curves showing rupture events and average number of rupture events per force curve, as acquired with CD44-modified probes with live B16-F10 cells treated without DTIC and treated with 25 and 75 μg/mL DTIC for 24 hours, respectively. Similar data were obtained using more than three different probes and independent samples.
Abbreviations: AFM, atomic force microscopy; BSA, bovine serum albumin; DTIC, dacarbazine.

I. Unbinding forces were detected on the border and apical regions .

II. The unbinding force for the CD44 ligand-receptor decreased slightly after treatment with DTIC.

III. The results of the AFM recognition experiment were consistent to the results of the LSCM and FCM assays.

IV. Treatment with different DTIC concentrations showed no significant decrease in CD44 unbinding force between CD44 ligand and receptor, while the CD44 nanodomains were reduced and became smaller.

Quantifying the change in kinetic and thermodynamic parameters

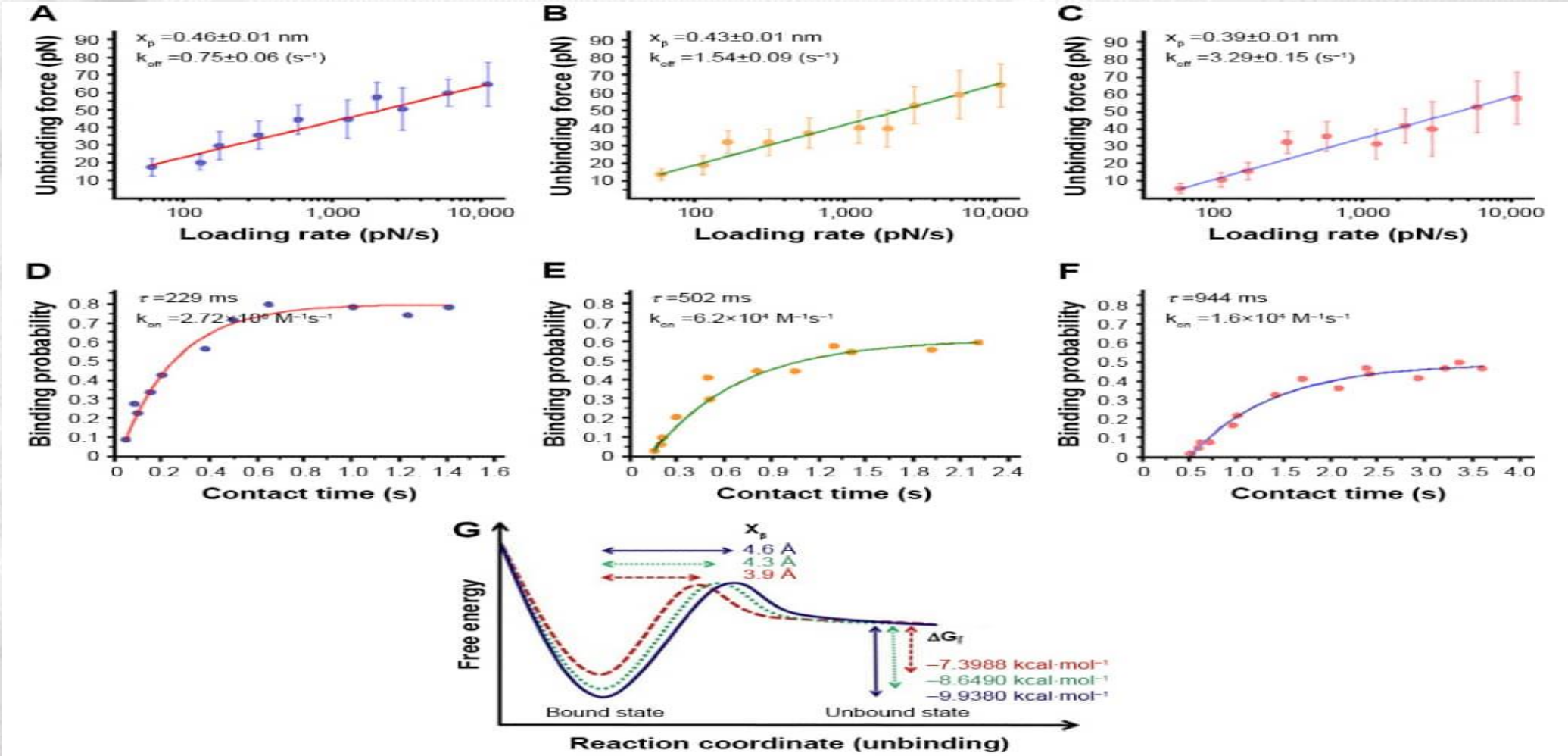


Figure 7 Quantifying the change in kinetic and thermodynamic parameters of single CD44 ligand–receptor bonds localized on B16-F10 cells in the absence and presence of DTIC under physiologically relevant conditions using dynamic force spectroscopy.

Notes: Loading-rate-dependent interaction forces between the CD44 tip and B16-F10 cells treated without DTIC (**A**) and treated with 25 (**B**) and 75 μg/mL DTIC (**C**) for 24 hours, respectively. The binding probability as a function of the dwell time of the CD44 tip on B16-F10 cells treated without DTIC (**D**) and treated with 25 (**E**) and 75 μg/mL DTIC (**F**) for 24 hours, respectively. (**G**) Free-energy binding landscape of the CD44 ligand in the absence and presence of DTIC. The blue line, green line, and red line represent control cells and cells treated with 25 and 75 μg/mL DTIC for 24 hours, respectively. x_β represents the distance from the energy minimum of the bound state to the transition state and is indicated for each group by different-colored horizontal arrows. ΔG gives the free-energy difference between the ligand-bound and unbound states and is indicated for each group by different-colored vertical arrows.

Abbreviation: DTIC, dacarbazine.

- After cells were treated with 25 and 75 micro molar per ml DTIC for 24 hours , the reconstructed CD44 ligand-binding free-energy decreased .

- These results demonstrate the CD44 ligand-receptor complexes on the surface of DTIC-treated melanoma cells to be less stable than those on the surface of untreated melanoma cells.

Conclusion:

AFM was used to investigate the effect of DTIC on binding strength and free-energy landscape

By using DTIC, new insights at the single molecule level were obtained for visual changes in CD44 nanodomain distribution and size

The CD44 ligand-receptor complexes on DTIC-treated cells were less stable than those on control cells

The dissociation constant increased and ligand-binding free-energy decreased when cells were treated

DTIC perturbs the kinetic & thermodynamic interaction of CD44 ligand binding but does not completely inhibit the binding of the CD44 ligand to its membrane receptor


THANK YOU